DEVELOPMENTAL BIOLOGY 179, 135–147 (1996)
ARTICLE NO. 0246

Nonmyristoylated MARCKS Complements Some but Not All of the Developmental Defects Associated with MARCKS Deficiency in Mice

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The myristoylated alanine-rich C kinase substrate, or MARCKS protein, is a widely expressed, prominent substrate for protein kinase C. Although the exact function of MARCKS has not been elucidated, targeted disruption of the MARCKS gene (*Macs*) in mice has shown that MARCKS plays a crucial role in the development of the central nervous system. Mice deficient in MARCKS exhibited universal perinatal death with defects in neurulation, fusion of the cerebral hemispheres, formation of the great forebrain commissures, and retinal and cortical lamination (Stumpo *et al., Proc. Natl. Acad. Sci. USA* 92, 944–948, 1995). In the present studies, a transgene consisting of approximately 3.4 kb of promoter from the human MARCKS gene (*MACS*), with an epitope tag sequence inserted at the carboxyl terminus of the MARCKS coding region, was able to complement completely MARCKS deficiency in mice. Thus, the human transgene contained all of the elements necessary for normal developmental expression of MARCKS. To test the importance of MARCKS myristoylation to its developmental role, an otherwise identical transgene was constructed in which the glycine at the amino terminus of MARCKS was mutated to an alanine. This mutation, which resulted in the expression of nonmyristoylated MARCKS, was successful in partially rescuing the *Macs* null phenotype. Specifically, about 25% of these mice survived the perinatal period; these survivors appeared to develop normally except for slightly decreased body size. In both the survivors and the nonsurvivors, all of the known anatomical defects associated with MARCKS deficiency were corrected by expression of the nonmyristoylated human protein. These results indicate that myristoylation of MARCKS is not required for the protein to correct many of the developmental abnormalities characteristic of its deficiency.

INTRODUCTION

A wide variety of cellular responses, including differentiation, mitogenesis, and hormone secretion, are mediated by the family of isoenzymes known collectively as protein kinase C (PKC) (Nishizuka, 1984, 1986, 1992; Goodnight *et al.*, 1994). However, little is understood about the mechanisms by which PKC controls these processes, or the precise roles of the phosphorylated substrates of PKC in mediating these events.

The myristoylated alanine-rich C kinase substrate, or MARCKS protein (for reviews see Aderem, 1992; Blackshear, 1993), is one of the most prominent of these cellular PKC substrates. It is characterized by amino-terminal myristoylation, in addition to heat stability and anomalous migration on SDS–polyacrylamide gels. There are three highly conserved regions in all MARCKS proteins identified to date: A consensus sequence for myristoylation at the amino-terminus which is in part responsible for the membrane association of the protein; a region of conserved sequence at the single site of intron splicing; and a phosphorylation site domain containing the three or four serines known to be phosphorylated by PKC. The phosphorylation site domain has also been shown to interact with calmodulin, actin, and cellular membranes, in all cases in a phosphorylation-inhibited manner.

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0012-1606/96 $18.00

To date, no precise function has been defined for MARCKS in cells. However, targeted disruption of the gene encoding MARCKS in mice (*Macs*) has implicated the protein in the development of the central nervous system and postnatal viability (Stumpo *et al.*, 1995). MARCKS-deficient mice exhibited universal perinatal death as well as high frequencies of exencephaly and omphalocele, failure of fusion of the cerebral hemispheres, agenesis of the corpus callosum and other forebrain commissures, and defects in cortical and retinal lamination. These defects point to a potential role for MARCKS in cell–cell interactions or cell migration during central nervous system development.

In the present studies, we have attempted to complement the developmental defects present in MARCKS-deficient animals through the expression of a transgene consisting of the intact human MARCKS gene (*MACS*) containing a hemagglutinin epitope tag sequence at the carboxyl-terminus. We show that this construct, which contains the entire human mRNA coding sequence as well as 3.4 kb of promoter and the single intron, is capable of fully complementing the MARCKS-deficiency phenotype in mice. We then investigated the importance of MARCKS myristoylation to its developmental role by attempting to rescue the *Macs* −/− mice with a transgene expressing nonmyristoylated human MARCKS. Our data indicate that the myristoyl modification of MARCKS is important for postnatal survival; 75% of the *Macs* −/− animals that expressed nonmyristoylated human MARCKS died in the immediate perinatal period. However, all of the known anatomical defects associated with MARCKS deficiency were corrected by expression of the nonmyristoylated human protein, and 25% of the animals expressing this protein survived. These results suggest that myristoylation of MARCKS is necessary for some but not all of the protein's functions during development.

MATERIALS AND METHODS

Construction of transgenic vectors MACS-12CA5 and MACS(A_2/G_2)-12CA5. A clone encompassing the human gene encoding MARCKS (*MACS*) was isolated as described (Harlan *et al.*, 1991). A 7.5-kb fragment was identified that hybridized to the human MARCKS cDNA; this fragment was isolated by agarose gel electrophoresis and further purified by Gene Clean (Bio 101, Inc., Vista, CA). This 7.5-kb restriction fragment was subcloned into the *Bam*HI site of Bluescribe (Stratagene, La Jolla, CA) to produce the clone *7.5 kb MACS* (Fig. 1A). Restriction fragment and sequencing analysis of the clone revealed the presence of approximately 3.4 kb of the *MACS* 5′ flanking region, as well as the entire mRNA coding sequence, the single intron, and approximately 0.6 kb of 3′ flanking region.

An epitope tag consisting of a 9-amino-acid peptide derived from influenza virus hemagglutinin (Kolodjiej and Young, 1991) was introduced in-frame between nucleotides 1289 and 1290 in the carboxyl terminal coding region of the *7.5 kb MACS* construct (Harlan *et al.*, 1991), five codons 5′ of the stop codon, yielding *MACS-12CA5* (Fig. 1B). Insertion of the epitope was achieved by site-directed mutagenesis (Amersham, Arlington Heights, IL), according to the manufacturer's protocol; its presence in frame was confirmed by dideoxy sequencing (Sanger *et al.*, 1977). This epitope is recognized by monoclonal antibody 12CA5 (Berkeley Antibody Co., Richmond, CA).

To generate the construct *MACS(A_2/G_2)-12CA5* (Fig. 1C), the amino-terminal glycine in the MARCKS protein was changed to an alanine using the Altered Sites oligonucleotide-directed *in vitro* mutagenesis kit from Promega (Madison, WI). Creation of the mutation was confirmed by dideoxy sequencing (Sanger *et al.*, 1977). Thus, this construct differs from *MACS-12CA5* in only two nucleotides, which result in the expression of epitope-tagged MARCKS that differed from MARCKS-12CA5 by only a single amino acid.

Transient transfection of LM/TK$^-$ cells. Mouse LM/TK$^-$ cells, which lack endogenous MARCKS mRNA and protein (Stumpo *et al.*, 1989), were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 m*M* glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and plated at a density of approximately 1.9×10^6 cells/100 mm plate. The following day, the cells were transfected with either *7.5 kb MACS* or *MACS-12CA5* using 200 μg/ml DEAE-dextran as described (Lopata *et al.*, 1984).

In vivo phosphorylation and immunoprecipitation of MARCKS proteins. Confluent plates of LM/TK$^-$ cells transiently transfected with 10 μg of either *7.5 kb MACS* or *MACS-12CA5* were serum-starved overnight in DMEM containing 1% bovine serum albumin (BSA, lyophilized and crystallized; Sigma, St. Louis, MO), 2 m*M* glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were labeled for 2 hr in 0.2 mCi/ml [*ortho*-^{32}P]phosphate as described (Blackshear *et al.*, 1986), followed by treatment with 1.6 μ*M* phorbol 12-myristate 13-acetate (PMA) in 0.01% (v/v) dimethyl sulfoxide, or the same concentration of dimethyl sulfoxide as a control, for 10 min. The cells were washed twice with ice-cold buffer containing 100 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* $MgSO_4$, 25 m*M* $NaHCO_3$, 10 m*M* Hepes, and 25 m*M* glucose, and harvested in 2 ml of lysis buffer as described (Spizz and Blackshear, 1996). The samples were then immunoprecipitated with either the polyclonal antibody to the amino-terminus of MARCKS (Lobaugh and Blackshear, 1990) or the monoclonal antibody 12CA5 as described (Spizz and Blackshear, 1996).

Generation of transgenic mice. The *Xba*I/*Xma*I fragments of *MACS-12CA5* (Fig. 1B) and *MACS(A_2/G_2)-12CA5* (Fig. 1C) containing epitope-tagged human MARCKS sequences were isolated by agarose gel electrophoresis and further purified using Gene Clean (Bio 101, Inc.). This DNA was used for injection into the pronuclei of fertilized mouse eggs from B6SJLF$_1$/J mice by standard procedures (Gordon *et al.*, 1980). Female mice expressing the *MACS-12CA5* transgene or the *MACS(A_2/G_2)-12CA5* transgene were identified by Southern blotting (see below), using a probe that distinguishes the transgene from the endogenous gene (Harlan *et al.*, 1991). These were then mated to C57BL/6J males heterozygous for the disrupted mouse *Macs* allele (Stumpo *et al.*, 1995). The resulting progeny that both expressed the *MACS-12CA5* transgene and were heterozygous for the disrupted *Macs* allele were mated to create mice that expressed the *MACS-12CA5* transgene, but were homozygous for the disrupted *Macs* allele. Similar mating strategies were employed with mice that expressed the *MACS(A_2/G_2)-12CA5* transgene and were heterozygous for the disrupted *Macs* allele.

Southern blot analysis. DNA was prepared from mouse tail

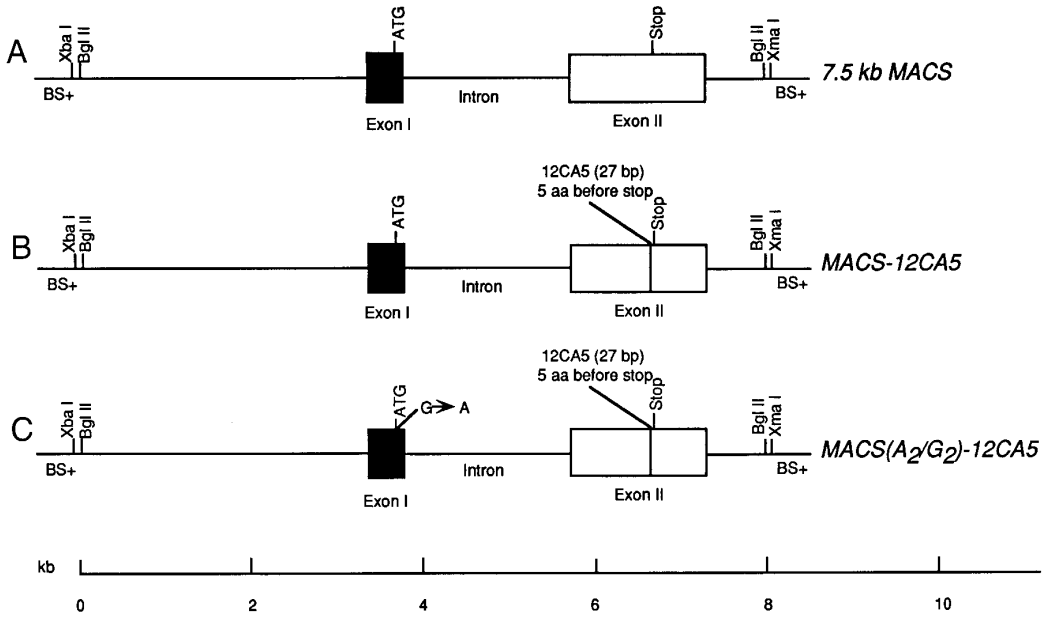

FIG. 1. Construction of transgenic vectors *MACS-12CA5* and *MACS(A_2/G_2)-12CA5*. A 7.5-kb *Bgl*II fragment isolated from a clone encoding human MARCKS (*MACS*) was subcloned into the *Bam*HI site of Bluescribe (BS^+; Stratagene) to produce *7.5 kb MACS* (A). An oligonucleotide encoding a 9-amino-acid peptide from influenza virus hemagglutinin was introduced in-frame between nucleotides 1289 and 1290 in the carboxy-terminal coding region of *7.5 kb MACS* (A) to yield *MACS-12CA5* (B). *MACS(A_2/G_2)-12CA5* (C) was created by changing the amino-terminal glycine of *7.5 kb MACS* (A) to an alanine by site-directed mutagenesis.

samples and digested with either *Eco*RI (detection of *MACS-12CA5* and *MACS(A_2/G_2)-12CA5* transgenes) or *Hin*dIII (detection of normal and disrupted *Macs* alleles), and separated on a 0.8% agarose gel. The DNA was transferred to Nytran (Schleicher & Schuell, Keene, NH) and probed with a 1.1-kb *Eco*RI fragment of the human genomic *MACS* clone (detection of *MACS-12CA5* and *MACS(A_2/G_2)-12CA5* transgenes) (Harlan *et al.*, 1991), or a 2.1-kb *Hin*dIII/*Sst*I fragment of the mouse genomic *Macs* clone (detection of normal and disrupted *Macs* alleles).

Preparation of mouse tissue homogenates. Organs from mice expressing the *MACS-12CA5* transgene were removed and rapidly placed in liquid nitrogen after the animals were euthanized with CO_2. The frozen tissues were ground to a fine powder under liquid nitrogen using a mortar and pestle. The resulting tissue powder was homogenized using a Polytron (Kinematica, GmbH, Lucerne, Switzerland) in 2 ml of an ice-cold buffer containing 50 m*M* β-glycerophosphate (pH 8.2), 250 m*M* sucrose, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* dithiothreitol, 10 m*M* benzamidine–HCl, 1 m*M* phenylmethylsulfonyl fluoride, 2 μ*M* pepstatin, 2 μ*M* leupeptin, and 0.6% Triton X-100. The homogenates were centrifuged at 4400*g* for 30 min at 4°C. The resulting supernatants were boiled for 10 min and centrifuged at 11,000*g* for 30 min at 4°C. The heat-stable proteins remaining in the supernatant were precipitated with 25% (w/v) trichloroacetic acid, washed with acetone (−20°C), and resuspended in phosphate-buffered saline (PBS) with 1/4 vol SDS sample buffer (1.5 *M* sucrose, 6% (w/v) SDS, 500 m*M* dithiothreitol, 60 m*M* EDTA, 0.006% (w/v) pyronin Y), boiled for 3 min, and subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting (see below).

Subcellular fractionation of mouse brain homogenates. Mouse brains were removed immediately after the animals were euthanized with CO_2, rinsed in ice-cold PBS, and homogenized using a Polytron (Kinematica) in 2–3 ml of the buffer described above for the preparation of mouse tissue homogenates, except that the Triton X-100 was not included. The resulting homogenates were subjected to ultracentrifugation at 107,000*g* for 40 min at 4°C (TLA 100.3 rotor, Beckman Instruments, Fullerton, CA). The supernatant fraction was removed, and the particulate fraction was resuspended in a volume of buffer equal to the starting volume that contained 0.6% Triton X-100 by sonication, and incubated on ice for 30 min with frequent resuspension. This membrane suspension was centrifuged at 16,000*g* for 15 min at 4°C, and the resulting supernatant was taken as the particulate fraction. Soluble and particulate fractions were then boiled for 10 min, centrifuged at 16,000*g* for 15 min at 4°C, and the supernatants containing the heat-stable proteins were combined with 1/5 vol SDS sample buffer, boiled for 3 min, and subjected to SDS–PAGE and Western blotting (see below).

Western blot analysis. Proteins from SDS–polyacrylamide gels were transferred to nitrocellulose and blocked in a solution of 3% (w/v) nonfat dry milk in TBS/T (10 m*M* Tris–HCl (8), 154 m*M* NaCl, 0.05% (v/v) Tween 20) at room temperature for 1 hr as previously described (Swierczynski and Blackshear, 1995). Blots were then incubated with either monoclonal antibody 12CA5, which recognizes the hemagglutinin epitope tag sequence (Berkeley Antibody Co., Richmond, CA) at a 1:800 dilution in TBS/T or a polyclonal antibody to an amino-terminal MARCKS peptide (Lobaugh and Blackshear, 1990). As a secondary antibody, either goat anti-mouse (for the 12CA5 monoclonal antibody) or goat anti-rabbit (for the polyclonal MARCKS antiserum) IgG conjugated to horseradish peroxidase (Bio-Rad, Richmond, CA) was used at a 1:5000 dilution

in TBS/T. Chemiluminescence (ECL kit, Amersham) was used for detection of immunoreactive proteins. For some experiments, color-reactive proteins were developed in a solution of TBS, 17% (v/v) methanol, 0.05% horseradish peroxidase color development reagent (Bio-Rad), and 0.015% (v/v) H_2O_2.

Histological analysis. Mouse tissues were removed and placed in Bouin's fixative (Polysciences, Inc., Warrington, PA) for 48 hr at room temperature. The fixed tissues were washed in 70% (v/v) ethanol at room temperature for several days, with several changes of the washing solution. The tissues were then embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin as previously described (Stumpo *et al.*, 1995).

Immunohistochemistry. For immunohistochemistry using the 12CA5 monoclonal antibody, embryos at Embryonic Day (E) 9.5 (where E 0.5 represents the date of vaginal plugging) were removed, and the genotype determined using the caudal half of the embryo as described above for tail blotting. The cranial halves of the embryos were fixed in 4% paraformaldehyde for 16 hr at 4°C, frozen in isopentane/acetone/dry ice, and then processed for frozen section immunohistochemistry essentially as previously described (LaMantia *et al.*, 1993). Sections (10 μm) were permeabilized with two 10-min washes in a TBS solution containing 0.2% Triton X-100; they were then incubated with 12CA5 at a 1:3000 dilution for 16 hr at 4°C and then stained with secondary antibody (FITC-labeled goat anti-mouse IgG; Calbiochem, La Jolla, CA) for 30 min at room temperature. Fluorescence microscopy and photography were performed as previously described (Blackshear *et al.*, 1996).

RESULTS

7.5 kb MACS and MACS-12CA5 are expressed in LM/TK$^-$ cells. In order to ensure that MARCKS proteins were expressed from constructs *7.5 kb MACS* and *MACS-12CA5*, we performed *in vivo* phosphorylation and immunoprecipitation experiments in transiently transfected LM/TK$^-$ cells; these cells ordinarily lack MARCKS mRNA and protein (Stumpo *et al.*, 1989). Cells transfected with *7.5 kb MACS* expressed a phosphoprotein that was recognized by the polyclonal antibody raised against the amino terminus of MARCKS (Lobaugh and Blackshear, 1990) (Fig. 2); similarly, cells transfected with *MACS-12CA5* expressed a phosphoprotein that was recognized by the antibody (12CA5) to the hemagglutinin epitope tag (Berkeley Antibody Co.) (Fig. 2). Both proteins exhibited the characteristic anomalous migration of human MARCKS on 7.2% SDS–PAGE gels, with apparent M_r of approximately 94×10^3.

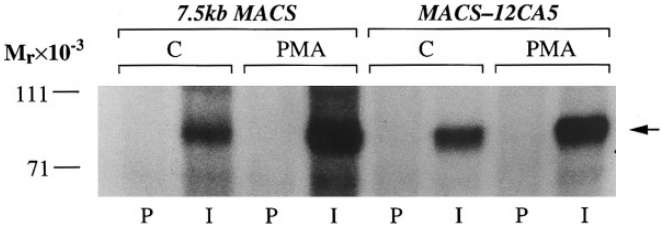

FIG. 2. Expression of *7.5 kb MACS* and *MACS-12CA5* transiently transfected in LM/TK$^-$ cells. LM/TK$^-$ cells, which do not express endogenous MARCKS, were transiently transfected with 10 μg of either *7.5 kb MACS* or *MACS-12CA5* DNA. The cells were serum-starved overnight and then labeled with 0.2 mCi/ml [*ortho*-^{32}P]-phosphate for 2 hr. Following treatment with control conditions (C) or 1.6 μM PMA for 10 min, the cells were homogenized and subjected to immunoprecipitation with preimmune serum (for *7.5 kb MACS;* P) or nonimmune ascites fluid (for *MACS-12CA5;* P), and either a polyclonal antibody raised against a MARCKS amino-terminal peptide (*7.5 kb MACS;* I) or a monoclonal antibody raised against the hemagglutinin epitope tag (*MACS-12CA5;* I). The resulting autoradiograph is shown. The arrow indicates the position of immunoprecipitated, phosphorylated MARCKS. The positions of molecular weight standards are shown.

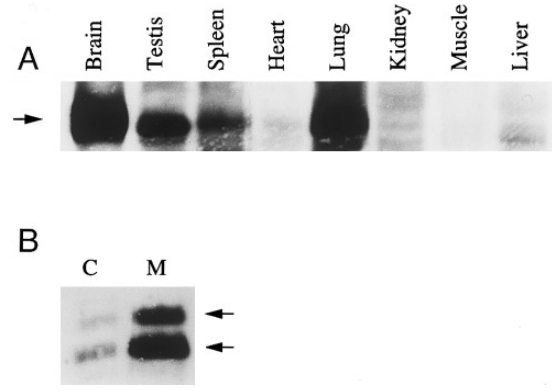

FIG. 3. Tissue-specific expression and subcellular fractionation of MARCKS-12CA5 in mice. Tissue extracts were prepared from a mouse expressing the *MACS-12CA5* transgene as described under Materials and Methods. The MARCKS proteins in the tissue samples were precipitated with 25% TCA, and equal volumes from each tissue, with the exception of brain where 25% of the volume was loaded, were subjected to SDS–PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody raised against the hemagglutinin epitope tag (A). The arrow indicates the position of epitope-tagged MARCKS. In B, brain homogenates were separated into membrane (M) and cytosolic (C) fractions by ultracentrifugation. The particulate fraction was resuspended in the original volume of homogenization buffer, and then equal volumes were subjected to SDS–PAGE, transferred to nitrocellulose, and probed with a polyclonal antibody raised against the amino-terminus of MARCKS. The top arrow indicates MARCKS-12CA5; the bottom arrow indicates endogenous mouse MARCKS.

3.4 kb of 5′-flanking MACS sequence promotes normal tissue distribution of human MARCKS. Of four lines of mice generated that expressed detectable human MARCKS DNA, two were selected for further analysis based on high level expression of the *MACS-12CA5* transgene. As determined by Western blot analysis of tissue homogenates prepared from various organs, the 3.4 kb of 5′-flanking region of *MACS* was sufficient to drive essentially normal tissue distribution of the MARCKS protein (Fig. 3A); i.e., the epitope-tagged protein was expressed at readily detectable lev-

els in brain, spleen, testis, and lung, and at somewhat lower levels in heart. These tissues normally express the highest levels of MARCKS in the mouse, as determined by Northern blot analysis (Lobach *et al.*, 1993). Expression of the epitope-tagged protein was minimal in liver, skeletal muscle, and kidney, as seen with the endogenous mRNA (Lobach *et al.*, 1993). The pattern of expression was similar in both lines of mice (data not shown).

In addition to the normal pattern of tissue-specific expression, the expressed, epitope-tagged human MARCKS protein was also primarily membrane-associated to approximately the same extent as the endogenous protein (Fig. 3B). Densitometric analysis of the Western blot shown in Fig. 3B indicated that 86% of the epitope-tagged protein was associated with the particulate fraction, with the remainder associated with the soluble fraction; 83% of the endogenous protein was membrane-associated (Fig. 3B). The marked difference in M_r of the human transgenic protein compared to the endogenous mouse protein allowed for the ready detection of both species in the same gel with the amino-terminal MARCKS antibody (Fig. 3B).

Expression of the MACS-12CA5 transgene results in full rescue of the Macs null phenotype. As reported earlier, targeted disruption of *Macs* in mice and complete MARCKS deficiency resulted in perinatal death associated with serious defects in the development of the central nervous system (Stumpo *et al.*, 1995). Because the epitope-tagged human protein exhibited a normal pattern of expression in the mouse, we attempted to rescue the phenotype of the null mutation with the *MACS-12CA5* transgene. Mice expressing both the *MACS-12CA5* transgene and one allele of disrupted *Macs* were mated to generate progeny that both expressed the transgene and were homozygous for disrupted *Macs;* this genotype was confirmed by Southern blot analysis. These mice were not only viable, but also appeared perfectly normal in size and development of the central nervous system, as described below. In addition, both sexes of *MACS-12CA5*-rescued *Macs* −/− mice were fertile, and females were able to sustain pregnancy with an average litter size of 4 ± 2.4 (SD) pups (n = 87 litters). Perinatal death of knockout pups not expressing the *MACS-12CA5* transgene may account for the small average litter size. To date, the oldest living rescued animals are 16 months of age.

Mice rescued with the MACS-12CA5 transgene exhibit normal brain development. Several defects in brain development are hallmarks of the MARCKS-deficient phenotype (Stumpo *et al.*, 1995). For example, *Macs* −/− animals displayed agenesis of the corpus callosum and other forebrain commissures, in addition to failure of fusion of the cerebral hemispheres. Coronal sections of brains from adult *MACS-12CA5*-rescued animals at approximately the level of the optic chiasm indicated that normal fusion of the cerebral hemispheres had taken place; in addition, the corpus callosum as well as the anterior and ventral hippocampal commissures were intact (Figs. 4A–4C).

A universal finding in the *Macs* −/− animals is a pronounced defect in cortical lamination, most easily seen as an irregular boundary between the cortical plate and the marginal zone in the developing forebrain (Stumpo *et al.*, 1995). Although the *MACS-12CA5*-rescued animals were evaluated in adulthood, when the cortical plate and marginal zone are no longer present in the neocortex, coronal sections displayed a normal boundary between the molecular layer (I) and the external granular layer in the frontal lobes (Figs. 5A–5C), indicating that cortical lamination was not impaired.

Macs −/− animals also displayed higher than normal frequencies of certain birth defects, specifically exencephaly (25%) and omphalocele (19%) (Stumpo *et al.*, 1995). Of 350 *MACS-12CA5*-rescued animals evaluated to date, no exencephaly or omphalocele has been observed.

Mice rescued with the MACS-12CA5 transgene exhibit normal retinal laminations. *Macs* −/− animals also exhibit lamination defects in the developing retina (Stumpo *et al.*, 1995), specifically a near-absence of the layer of Chievitz, which separates the inner and outer nuclear layers in fetal mice and neonatal mice (Chievitz, 1887; Smelser *et al.*, 1973). Although the *MACS-12CA5*-rescued animals were evaluated in adulthood, when the layer of Chievitz is no longer present in the retina, retinas from these animals exhibited totally normal patterns of retinal layering (Figs. 6a–6c).

Subcellular fractionation of nonmyristoylated human MARCKS expressed in transgenic animals. Of five lines of mice generated that expressed detectable *MACS(A_2/G_2)-12CA5* DNA, three lines were selected for further analysis based on high level expression of nonmyristoylated human MARCKS protein. Animals expressing the *MACS(A_2/G_2)-12CA5* transgene appeared perfectly normal and did not exhibit any deleterious dominant negative effects caused by high-level expression of the nonmyristoylated MARCKS protein. These three lines of mice were used to determine the subcellular localization of nonmyristoylated human MARCKS expressed in transgenic animals. Upon fractionation of brain extracts by ultracentrifugation, nonmyristoylated human MARCKS was associated with both the soluble and particulate fractions (Fig. 7A). Densitometric analysis of the Western blot from the three different lines of mice shown in Fig. 7A indicated that approximately 51, 57, and 52% (mean = 53%) of the nonmyristoylated, epitope-tagged protein was associated with the particulate fraction. This should be compared to the distribution of the endogenous, fully myristoylated mouse protein evaluated in the same blot, in which essentially all of the protein was associated with the particulate fraction (Fig. 7A). These results indicate that, in contrast to both the endogenous mouse protein and the transgenic wild-type human protein, a significant portion (40–50%) of nonmyristoylated MARCKS was associated with the soluble fraction prepared from brain extracts.

Immunohistochemistry of transgenic normal and nonmyristoylated MARCKS. The antibody 12CA5 was used to evaluate the subcellular distribution of transgenic human MARCKS, either wild-type or nonmyristoylated, in the devel-

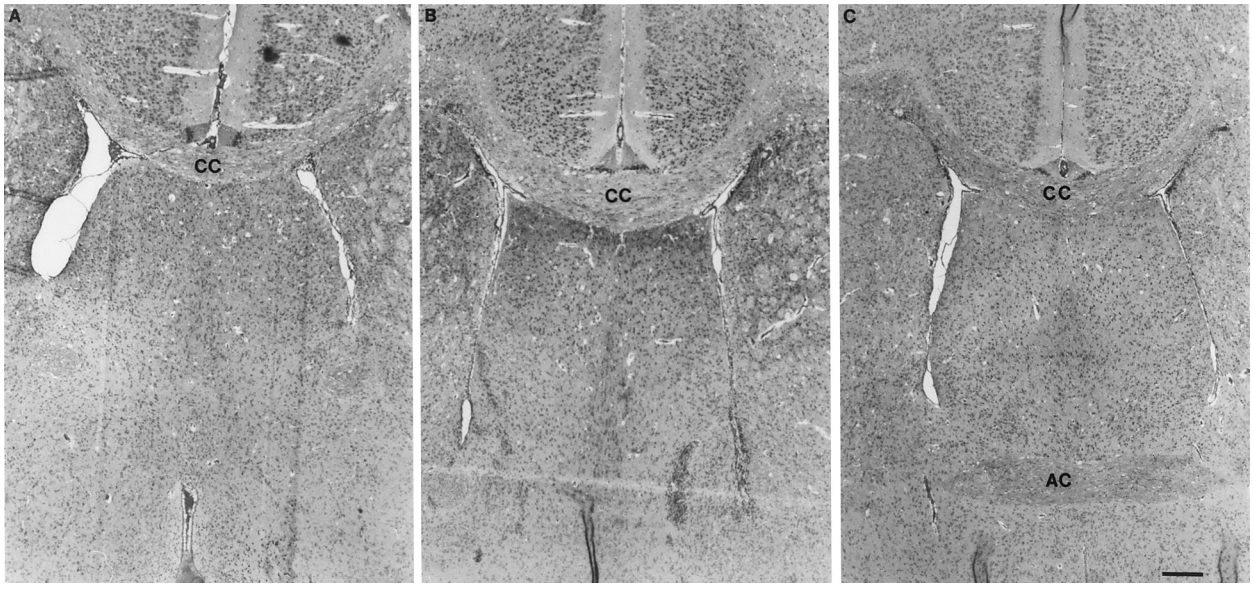

FIG. 4. Corpus callosum in *MACS-12CA5*-rescued animals. Portions of hematoxylin- and eosin-stained coronal sections of brains from an adult wild-type or *Macs* +/+ mouse (A) and two adult *MACS-12CA5*-rescued, *Macs* −/− animals (B, C), all from the same litter, at approximately the level of the optic chiasm are shown. CC, corpus callosum; AC, anterior commissure. Bar, 200 μm.

oping brains of E 9.5 transgenic mice. This early date was chosen because endogenous MARCKS can be localized clearly to the plasma membrane of individual cells at this time (Blackshear *et al.*, 1996). Epitope-tagged human MARCKS was clearly associated with the plasma membrane of the neuroepithelial, surface ectoderm, and mesenchymal cells (Fig. 8), a pattern indistinguishable from that seen with endogenous mouse MARCKS (Blackshear *et al.*, 1996). In contrast, the nonmyristoylated human protein was clearly localized, at least in part, to the cytoplasm of all three types of cells (Fig. 8). The specificity of the antibody was indicated by the complete lack of staining of tissues from normal, nontransgenic littermate embryos processed in parallel (Fig. 8).

Expression of the MACS(A_2/G_2)-12CA5 transgene fails to rescue completely the MARCKS null phenotype in 75% of animals. In order to determine if the absence of the myristoyl moiety would have any adverse effects on the function of the MARCKS protein in development, we also attempted to rescue the null phenotype with mice expressing the *MACS(A_2/G_2)-12CA5* transgene. Expression of this mutant gene product was at least as great as that of the normal transgene product in whole brain homogenates (Fig. 7B). To date, 75% of mice with the appropriate ''rescue'' genotype (n = 28 total whose genotype could be determined with certainty) died before or shortly after birth, similar to the *Macs* −/− mice (Stumpo *et al.*, 1995). These animals appeared normal morphologically, but were never observed to have much milk in the stomach. However, these animals did not exhibit any of the major histological defects in central nervous system development associated with the *Macs* null phenotype. Every newborn mouse that expressed the *MACS(A_2/G_2)-12CA5* transgene and was homozygous for the disrupted *Macs* gene exhibited normal fusion of the cerebral hemispheres and formation of the corpus callosum and the anterior and ventral hippocampal commissures (Fig. 9). These animals also displayed normal patterns of cortical lamination, as evidenced by the smooth boundary between the marginal zone and the cortical plate in the neocortex (Fig. 10), and normal retinal layering, as evidenced by the presence of a well-developed layer of Chievitz (Fig. 11). No instances of exencephaly or omphalocele have been observed in these mice, in contrast to the high frequencies (25 and 19%, respectively) of these defects in the *Macs* −/− mice (Stumpo *et al.*, 1995). Newborn mice that were homozygous for the disrupted *Macs* gene, but did not express the *MACS(A_2/G_2)-12CA5* transgene, exhibited the characteristic histological abnormalities associated with the *Macs* −/− phenotype (data not shown).

Interestingly, 25% of mice with the appropriate ''rescue'' genotype (7 of 28) were viable. To date, these surviving animals appear healthy and behaviorally and phenotypically normal, except that they weigh, on average, about 23% less than their littermates (Fig. 12). As determined by densitometric analysis of the Southern blots, the relative amounts of the transgene in the viable and nonviable animals were not significantly different.

DISCUSSION

These studies have demonstrated that expression of an epitope-tagged, human MARCKS protein in *Macs* −/− ani-

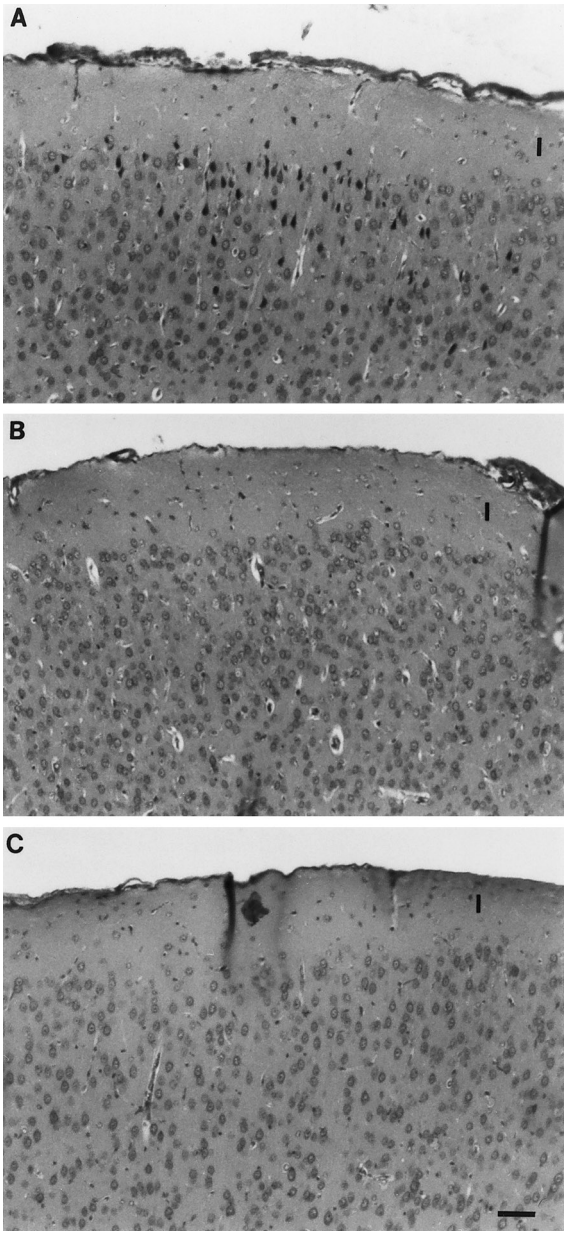

FIG. 5. Cortical laminations in *MACS-12CA5*-rescued animals. Hematoxylin- and eosin-stained sections of neocortex from brains of the same littermates shown in Fig. 4, i.e., a wild-type *Macs* +/+ mouse (A) and two *MACS-12CA5*-rescued, *Macs* −/− animals (B, C), are shown. I, molecular layer. Bar, 50 μm.

mals resulted in full rescue of the neurodevelopmental defects associated with the *Macs* −/− phenotype (Stumpo *et al.*, 1995). Mice expressing the MARCKS-12CA5 protein, driven by 3.4 kb of the *MACS* promoter, exhibited intact forebrain commissures, fusion of the cerebral hemispheres, and normal cortical and retinal laminations. They also exhibited normal rates of perinatal survival, and no instances of exencephaly or omphalocele were detected. Finally, these transgenically rescued animals appeared normal in every way, including normal size and fertility. Because the *Macs* −/− animals examined in our previous studies were derived from a single line of ES cells (Stumpo *et al.*, 1995), the possibility existed that the observed defects were not due simply to MARCKS deficiency. However, complete rescue of the *Macs* −/− phenotype with the *MACS-12CA5* transgene confirms that MARCKS plays an integral role in the development of the central nervous system, and that the previously observed defects (Stumpo *et al.*, 1995) were indeed the result of MARCKS deficiency alone. Complete rescue of the *Macs* −/− phenotype with the MARCKS-12CA5 protein also demonstrates that expression of the 9-amino-acid hemagglutinin epitope tag at the carboxyl-terminus of MARCKS had no deleterious effects on the normal developmental function of the protein.

Complete rescue of the *Macs* −/− phenotype with the *MACS-12CA5* transgene also indicates that the 3.4 kb of upstream *MACS* sequences was sufficient to direct normal spatial and temporal expression of the MARCKS-12CA5 protein during development. Previous studies examining the promoter regions of human (Harlan *et al.*, 1991) and mouse (Blackshear *et al.*, 1992) MARCKS have shown that the 5′-flanking regions are highly conserved (89% identical) between the two species. Both promoters lack a TATA box and include potential binding sites for transcription factors SP1 and CTF/NF-1 (Mitchell and Tjian, 1989). In transient transfections in LM/TK$^-$ cells, 248 bp of upstream sequences was sufficient to drive normal expression of a human growth hormone construct, whereas a construct containing 110 bp of upstream sequences was essentially inactive (Harlan *et al.*, 1991). Although the *MACS* promoter has not been characterized further, the present replacement experiments indicate that 3.4 kb of upstream sequence, combined with the endogenous intron and 3′-untranslated region, contains all of the elements necessary for normal MARCKS expression in the intact animal during development.

These studies thus form the basis for experiments designed to test the importance in development of specific sequence elements within the MARCKS protein. In the first such test of ''structure–function'' relationships, we attempted to rescue the null phenotype with a transgenic construct that was identical in every way to the construct that successfully complemented the null phenotype, except for changes in two nucleotides that led to the $gly_2 \rightarrow ala_2$ mutation, resulting in a nonmyristoylatable protein . Expression of this mutant protein in brain was at least as great as that of the wild-type human protein that corrected the null phenotype, as determined by immunoblotting. The nonmyristoylated status of the protein was reflected in its approximately 50% representation in the brain cytosolic fraction, compared with 14% of the normal human protein. Although about 75% of the *Macs* −/− pups expressing this

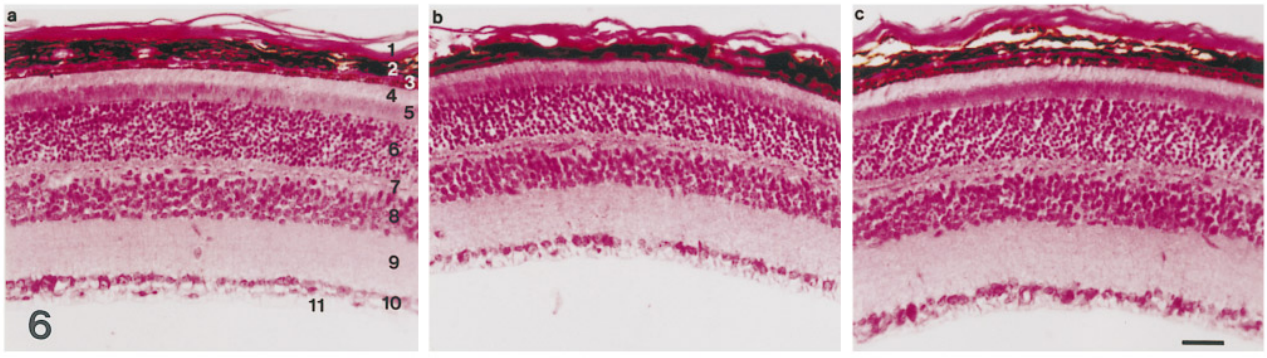

FIG. 6. Retinal layering in *MACS-12CA5*-rescued animals. Hematoxylin- and eosin-stained sections of retinas from the same littermates shown in Figs. 4 and 5, i.e., a wild-type *Macs* +/+ mouse (a) and two *MACS-12CA5*-rescued *Macs* −/− animals (b, c), are shown. 1, sclera; 2, choroid plexus; 3, retinal pigment epithelium; 4, rod outer segment; 5, rod inner segment; 6, outer nuclear layer; 7, outer plexiform layer; 8, inner nuclear layer; 9, inner plexiform layer; 10, ganglion cell layer; 11, retinal ganglion cell axons. Bar, 25 μm.

FIG. 12. Phenotype of MARCKS-deficient mice expressing the *MACS(A_2/G_2)-12CA5* transgene. Two mice of the *Macs* −/− genotype expressing the *MACS(A_2/G_2)-12CA5* transgene (ko-r) and a littermate of the *Macs* +/− genotype expressing the *MACS(A_2/G_2)-12CA5* transgene (wt) at 3 weeks of age are shown. Their body weights were 8 g (for both ko-r mice) and 12 g (wt).

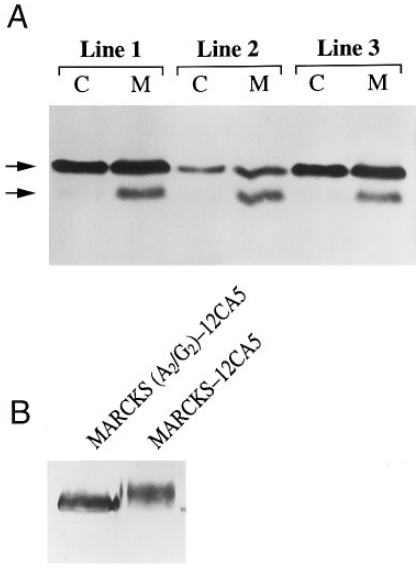

FIG. 7. Subcellular fractionation of MARCKS (A_2/G_2)-12CA5 in brain extracts. Brain homogenates from individual adult mice representing three separate (1–3) lines of transgenic animals expressing MARCKS(A_2/G_2)-12CA5 were separated into membrane (M) and cytosolic (C) fractions by ultracentrifugation. The membrane fraction was resuspended in the original volume of homogenization buffer, and then equal volumes were subjected to SDS–PAGE, transferred to nitrocellulose, and probed with a polyclonal antibody raised against a MARCKS amino-terminal peptide (A). The top arrow points to the nonmyristoylated human protein; the bottom arrow points to the endogenous mouse protein. In B, brain homogenates from adult mice expressing nonmyristoylated human MARCKS (MARCKS (A_2/G_2)-12CA5) and wild-type human MARCKS (MARCKS-12CA5) were subjected to SDS–PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody raised against the hemagglutinin epitope tag.

mutant protein did not survive the perinatal period, the 25% that did were viable and phenotypically normal, although modestly decreased in size relative to their littermates. The females appear to be normal with respect to reproductive behavior and fertility. In addition, none of the rescued animals ($n = 28$) displayed any of the gross anatomical defects characteristic of the *Macs* −/− phenotype (Stumpo *et al.*, 1995). That the presence of MARCKS protein sequences per se is not sufficient to complement the MARCKS deficiency phenotype is suggested by the fact that an otherwise identical transgene expressing a MARCKS–β-gal fusion protein is totally ineffective at rescuing any aspects of the null phenotype (S. L. Swierczynski, W. S. Lai, and P. J. Blackshear, submitted for publication). Therefore, we can conclude from these experiments that the myristoyl modification of the MARCKS protein is not necessary for many of the developmental processes disrupted in the −/− mice, or for the postnatal survival of at least a substantial fraction of these animals. The ability of a subset of these animals to survive is most likely a stochastic phenomenon, in that viable animals occurred randomly throughout the population. Experiments currently underway involving intercrosses of the rescued animals should help us to determine the true frequency of survivors in a large population, and whether survival is random or implicates potential modifier genes.

To our knowledge, this is the first report of an attempt to rescue a null mutation in mice with the nonmyristoylated form of a protein. However, complementation studies have been performed with nonmyristoylated proteins in the budding yeast, *Saccharomyces cerevisiae* (Johnson *et al.*, 1994). Myristoylation was shown to be essential for the proper function of both the ADP-ribosylation factor, Arf1 (Kahn *et al.*, 1995), and the pheromone-responsive G protein α subunit, Gpa1 (Stone *et al.*, 1991). Yeast cells expressing nonmyristoylated Arf1 were not viable (Kahn *et al.*, 1995), and cells expressing nonmyristoylated Gpa1 exhibited constitutive activation of the mating pathway (Stone *et al.*, 1991). On the other hand, a nonmyristoylated form of the Vps15 protein kinase behaved essentially like wild-type Vps15 in yeast, although it was phosphorylated to a lesser extent (Herman *et al.*, 1991).

In higher eukaryotic cells, mutations preventing N-myristoylation have been shown to affect the function of many proteins. For example, nonmyristoylated forms of the gag polyprotein precursors from human immunodeficiency virus (Bryant and Ratner, 1990), Moloney murine leukemia virus (Rein *et al.*, 1986), and Mason–Pfizer monkey virus (Rhee and Hunter, 1987) were defective in assembly of the mature virion and the production of infectious particles. Myristoylation was also required for $p60^{src}$ (Cross *et al.*, 1984; Buss *et al.*, 1986) and $p56^{lck}$ (Abraham and Veillette, 1990) to transform cells. In addition, myristoylation was necessary for $p60^{src}$ to serve as a substrate for protein kinase C (Buss *et al.*, 1986). Nonmyristoylated ADP-ribosylation factor 6 had no effect on internalization and recycling of the transferrin receptor (D'Souza-Schorey and Stahl, 1995), indicating that myristoylation was required for this protein to play its normal role in endocytic trafficking.

For other myristoyl proteins, myristoylation is required for only a subset of their functions. For example, the catalytic subunit of cAMP-dependent protein kinase does not require myristate for holoenzyme formation (Clegg *et al.*, 1989; Yonemoto *et al.*, 1993), activation of the enzyme (Clegg *et al.*, 1989; Slice and Taylor, 1989; Yonemoto *et al.*, 1993), regulation of cell morphology (Clegg *et al.*, 1989), synthesis of steroids (Clegg *et al.*, 1989), or regulation of the expression of cellular genes (Clegg *et al.*, 1989). Instead, myristoylation appears to be required for the structural stability of the catalytic subunit (Clegg *et al.*, 1989; Slice and Taylor, 1989; Yonemoto *et al.*, 1993; Zheng *et al.*, 1993). Nonmyristoylated $p60^{src}$ exhibits tyrosine kinase activity (Buss *et al.*, 1986), yet displays the defects discussed above.

Previous studies of MARCKS myristoylation have

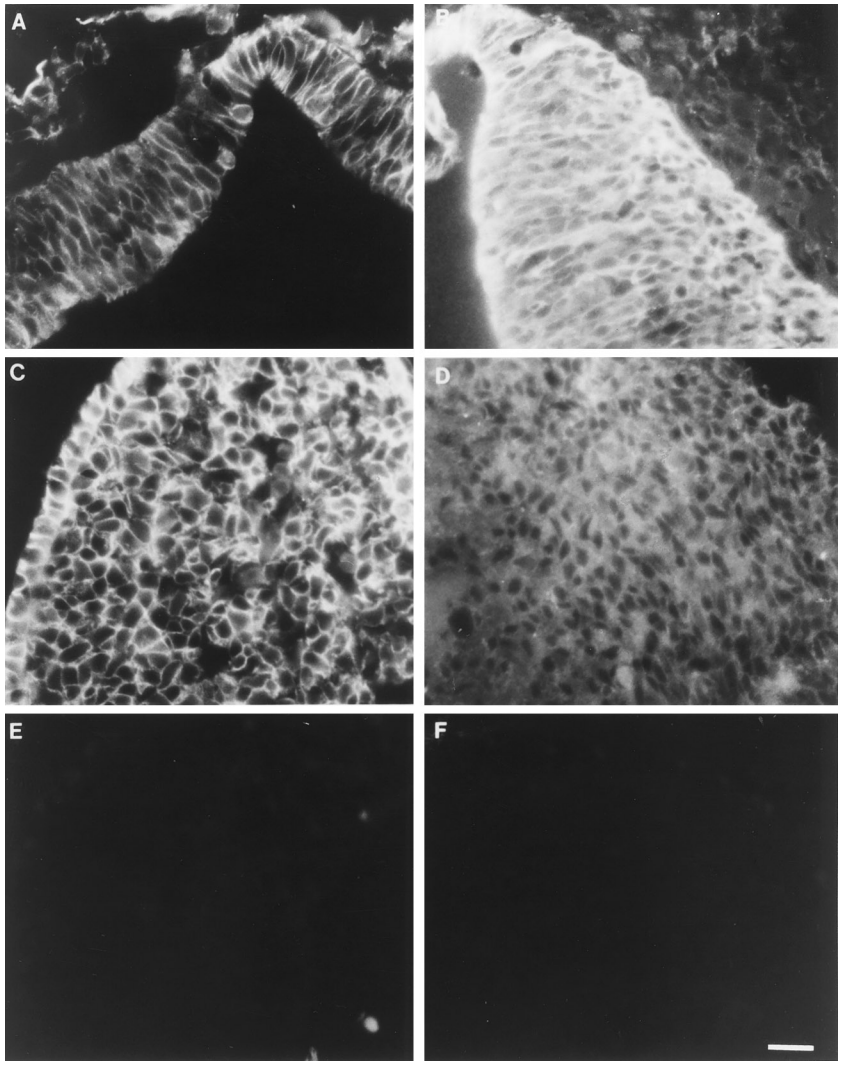

FIG. 8. Immunohistochemistry of coronal sections from E 9.5 mice expressing the *MACS-12CA5* and *MACS(A_2/G_2)-12CA5* transgenes. Coronal sections of brains from E 9.5 mice of the *Macs* −/− genotype expressing the *MACS-12CA5* transgene (A, neural tube; C, mesenchyme), the *Macs* +/+ genotype expressing the *MACS(A_2/G_2)-12CA5* transgene (B, neural tube; D, neural tube), and the *Macs* +/+ genotype expressing no transgene (E, neural tube) incubated with the monoclonal antibody 12CA5 are shown. (F) A coronal section from the mouse with the *Macs* +/+ genotype expressing the *MACS(A_2/G_2)-12CA5* transgene that was not incubated with the 12CA5 antibody, but was otherwise processed in parallel with the other sections. As a secondary antibody for all sections, goat anti-mouse IgG conjugated to fluorescein isothiocyanate was used. Bar, 25 μm.

demonstrated that myristoylation is necessary for the full extent of MARCKS membrane association. For example, in cell-free assays, nonmyristoylated MARCKS exhibited markedly decreased affinity for fibroblast membranes when compared to the fully myristoylated protein (Swierczynski and Blackshear, 1995). In cell expression studies, about 40% of the nonmyristoylated protein was associated with the plasma membrane, compared to 80% of the wild-type protein. In the present study, about 50% of the nonmyristoylated protein was still associated with a brain membrane fraction, compared to 80% of the wild-type protein. Nonetheless, nonmyristoylated MARCKS can still serve as a substrate for PKC in intact cells (Graff *et al.*, 1989). In addition, the polybasic phosphorylation site domain has been shown to exert an independent membrane targeting effect, both in cell-free systems and in intact cells (Kim *et al.*, 1994a,b; Swierczynski and Blackshear, 1995, 1996). Therefore, it may be that enough of the nonmyristoylated protein can associate with the plasma membrane in these rescued animals to subserve

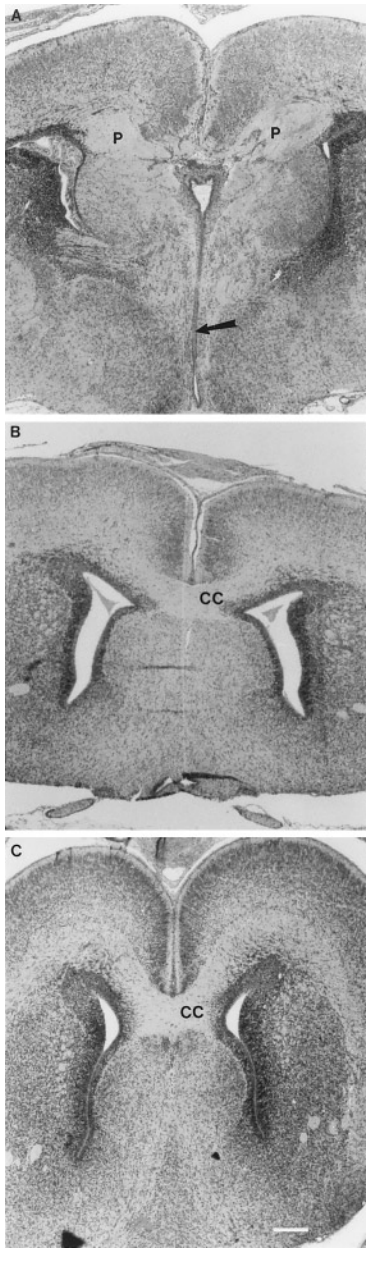

FIG. 9. Coronal sections from newborn MARCKS-deficient mice expressing the *MACS(A_2/G_2)-12CA5* transgene. Hematoxylin- and eosin-stained coronal sections of brains from newborn mice of the *Macs* −/− genotype (A), *Macs* +/+ genotype (B), and the *Macs* −/− genotype expressing the *MACS(A_2/G_2)-12CA5* transgene (C) are shown. The arrow indicates the persistent interhemispheric fissure in the −/− mouse; note also the absence of the corpus callosum (CC) and the presence of Probst's bundles (P) in A. Bar, 200 μm.

many, if not all, of MARCKS's developmental functions. Future studies will address the question of whether a nonmyristoylatable protein in which the four serines phosphorylated by PKC have been mutated to aspartates can complement any aspects of the null phenotype; this double mutant protein has essentially no membrane af-

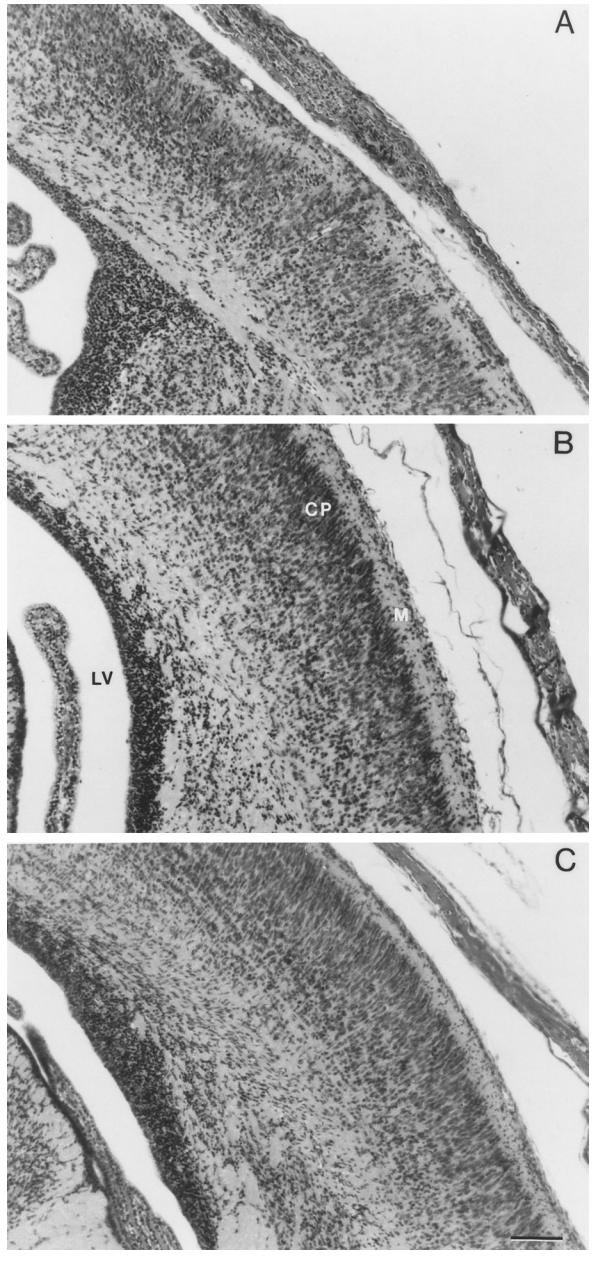

FIG. 10. Sections of lateral cortex from newborn MARCKS-deficient mice expressing the *MACS(A_2/G_2)-12CA5* transgene. Hematoxylin- and eosin-stained sections of the neocortex from newborn mice with the *Macs* −/− genotype (A), *Macs* +/+ genotype (B), and the *Macs* −/− genotype expressing the *MACS(A_2/G_2)-12CA5* transgene (C) are shown. Note the jagged boundary between the cortical plate and the marginal zone in A, which is smooth in B and C. M, marginal zone; CP, cortical plate; LV, lateral ventricle. Bar, 100 μm.

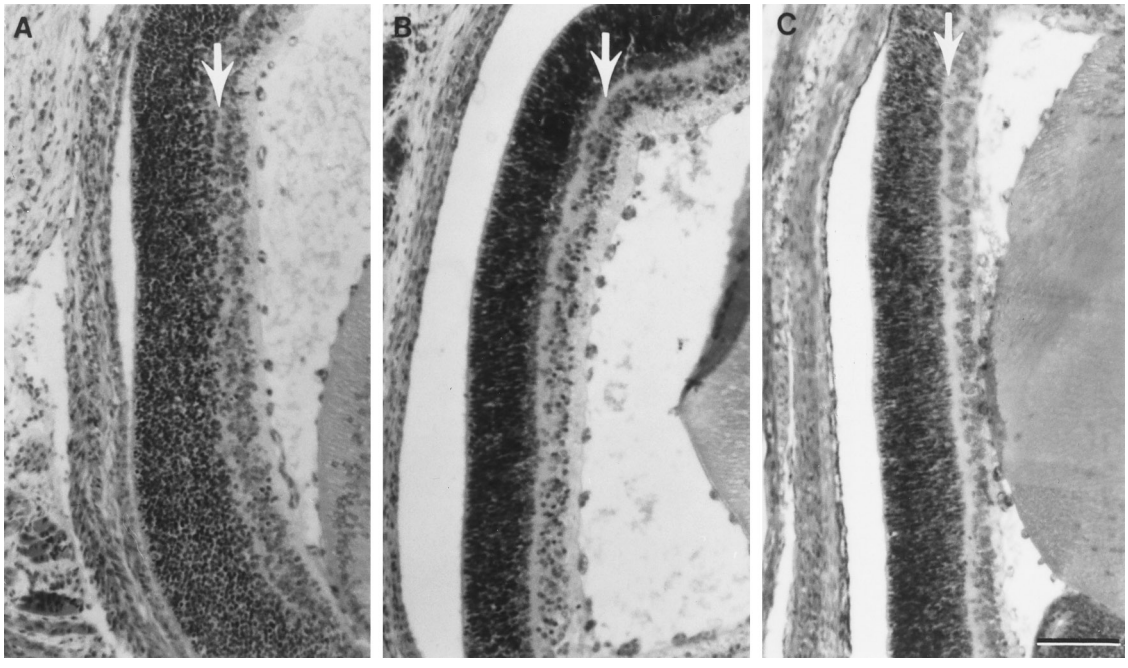

FIG. 11. Retinal sections from newborn MARCKS-deficient mice expressing the *MACS(A_2/G_2)-12CA5* transgene. Hematoxylin- and eosin-stained sections of retina from newborn mice of the *Macs* −/− genotype (A), *Macs* +/+ genotype (B), and the *Macs* −/− genotype expressing the *MACS(A_2/G_2)-12CA5* transgene (C) are shown. The arrow indicates the transient layer of Chievitz, which is nearly absent in A but is well-defined in B and C. Bar, 100 μm.

finity, either in cell-free assays (Swierczynski and Blackshear, 1995) or in cultured cells (Swierczynski and Blackshear, 1996).

ACKNOWLEDGMENTS

We thank Cheryl Bock and Wendy Callahan of the Transgenic Mouse Shared Facility of the Duke Comprehensive Cancer Center for creating the transgenic mice. We also thank Deborah Stumpo for supplying the *Macs* +/− mice. S.L.S. was supported in part by the Howard Hughes Medical Institute Graduate Student Support Fund. P.J.B. is an Investigator of the Howard Hughes Medical Institute.

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Received for publication June 10, 1996
Accepted July 29, 1996